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Platelets play a central role in sensitization to allergen.

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Abstract

Background: Platelet activation occurs in patients with allergic inflammation, and platelets can be activated directly by allergen via an IgE-dependent process. Platelets have been shown to activate antigen-presenting cells (APCs) such as CD11c⁺ dendritic cells (DCs) *in vitro*. Whilst CD11c⁺ DCs are requisite for allergen sensitization, the role of platelets in this process is unknown.

Objectives: We investigated whether platelets were necessary for allergen sensitization.

Methods: Balb/c mice sensitised to ovalbumin (OVA), were exposed to subsequent aerosolized allergen (OVA challenge). We analysed lung CD11c⁺ cell activation, co-localization with platelets, and some other indices of inflammation. The role of platelets at the time of allergen sensitization was assessed through platelet depletion experiments restricted to the period of sensitization.

Results: Platelets co-localized with airway CD11c⁺ cells, and this association increased after allergen sensitization, and after subsequent allergen exposure. Temporary platelet depletion (>95%) at the time of allergen sensitization led to a suppression of IgE and IL-4 synthesis, and a decrease in the pulmonary recruitment of eosinophils, macrophages, and lymphocytes after subsequent allergen exposure. Furthermore, in mice previously depleted of platelets at the time of sensitization, the recovered platelet population were shown to have reduced expression of FcεRI. Pulmonary CD11c⁺ cell recruitment was suppressed in these mice after allergen challenge, suggesting the migration of CD11c⁺ cells *in vivo* may be dependent on direct platelet recognition of allergen.

Conclusion: Platelets are necessary for efficient host sensitization to allergen. This propagates the subsequent inflammatory response during secondary allergen exposure, and increases platelet association with airway CD11c⁺ cells.

Keywords: Platelets, allergen sensitization, FcεRI, IgE, IL-4, CD11c, dendritic cells, leukocyte recruitment, asthma

List of abbreviations used

APC, antigen presenting cell

BALF, broncho-alveolar lavage fluid

DCs, Dendritic cells

OVA, Ovalbumin

Introduction

Platelet activation in allergic diseases such as asthma, has been reported extensively over the last 30 years, with evidence of changes in platelet volume, presence of platelet micro-particles, release of platelet-specific inflammatory mediators, and an increased incidence of circulating platelet-leukocyte (including eosinophil) complexes, both in patients undergoing spontaneous asthma attacks or after allergen challenge.¹⁻⁴ Furthermore, platelet activation correlates strongly to changes in lung function.⁴⁻⁹ Various *in vivo* studies confirm that the activation of platelets during allergy and asthma plays a central role in the inflammatory response.^{3,10-13} Of significance, pulmonary platelet recruitment and accumulation occurs in the lungs, initially via an IgE-dependent process whereby platelets migrate and enter the extravascular space.^{7,14-17} The significance and temporal nature of allergen-induced activation of platelets, via FcεRI, has not been established, yet of pertinence to allergy, platelets from patients allergic to *Dermatophagoides pteronyssinus* have been shown to be activated directly by the specific allergen, or by synthetic peptides derived from the allergen, through an IgE dependent process.^{16,18} Combined, these studies argue for a causal role of platelet activation in asthma and allergy, and that such activation is not as a consequence of the inflammatory milieu *per se*. Furthermore, it has been shown that platelet-derived 5-HT is also important for dendritic cell (DC) activation during allergic airways inflammation, further supporting a role for platelets, and suggesting that the activation and migration of platelets induced by allergen might be of significance or causal to the sensitization process.¹⁹ Myeloid DCs (mDCs) are thought to be involved very early in the development of asthma by inducing sensitization to inhaled antigen, and increasingly, mDCs are viewed as essential pro-

inflammatory cells that induce effector cytokine production in primed Th2 cells during airway inflammation.²⁰⁻²³ It is therefore of interest that platelet CD40L has recently been reported to be involved in the promotion of allergic airways inflammation by polarizing Th2 responses *after* allergen exposure.²⁴ We have previously demonstrated a presence of platelets extra-vascularly in the lungs of mice before subsequent allergen exposure in allergen-sensitized mice, but the vast majority of these platelets were attached to a resident undefined mononuclear cell population, and the relevance of this occurrence has yet to be established.¹⁶ We hypothesized that these platelets are a distinct population that had migrated to the lungs as a result of exposure to allergen (ip injection of ovalbumin, OVA), and that a population of platelets might therefore be involved in initial sensitization to allergen. Temporally, the occurrence was clearly unrelated to subsequent aerosolized exposures to allergen (OVA), which drives platelet migration into the lungs along with recruited leukocytes.¹⁶ We report here that CD11c⁺ cells (as a marker for DCs) are decorated with platelets during allergen sensitization, and investigate the importance of these platelets during the process of allergen sensitization and their interactions with CD11c⁺ cells, before and during subsequent exposure to aerosolized allergen. These studies provide a possible role for the specific activation of platelets by allergen from patients with asthma, and the occurrence of platelets in the lungs of patients with asthma.

121

122 **Materials and Methods**

123 Readers are directed to the online supplementary material document for methodology related
124 to: Blood microsampling, Immunohistochemistry of lungs, Microscopic analysis and lung
125 morphometry, Flow cytometric analysis of CD11c+ cells and platelets, and: Quantification of
126 IgE, IL-4, IL-5, and IFN- γ .

127

128 *1. Sensitisation of mice to ovalbumin and bronchoalveolar lavage (BAL).*

129 To understand the importance of platelets during allergen sensitization, and subsequent effects
130 on the inflammatory response after secondary allergen exposure via an aerosol challenge, a
131 murine model of allergic sensitization and lung inflammation was used where the sensitization
132 process can be delineated and confined by a defined route of administration (intraperitoneal
133 route) compared to the more complex situation arising from continual exposure to allergen via
134 the airways. All studies were carried out under the Animals (Scientific Procedures) Act of 1986,
135 and amendments 2012 (United Kingdom) with local ethical approval from King's College,
136 London. Studies and reporting have been undertaken under the ARRIVE guidelines. Female
137 Balb/c mice (20-25g; Harlan UK), housed under standard husbandry conditions of 12 hour
138 light/dark cycle, 21-23°C, in groups of 4-6 animals per cage with access to pelleted chow diet
139 and water *ad libitum*, were randomly assigned to different treatment groups. Mice were
140 sensitised with chicken egg ovalbumin (OVA, grade V, Sigma-Aldrich; 10 μ g/0.4ml, absorbed in a
141 saturated solution of an adjuvant: aluminium hydroxide, 0.1M, Sanofi Winthrop), or received

adjuvant only (sham-sensitized mice), intraperitoneally (i.p.) on days 0 and 7. On days 15, 16, and 17, mice were subsequently nebulised with OVA (10mg/ml) for 30 minutes, 24 hours post final allergen challenge (day 18). BAL fluid was processed for total and differential cell counts, and the enumeration of cytokines, whilst blood was processed for measurement of total IgE content as previously described.^{10,25} Some groups of mice were sacrificed 0, 24, and 48h after one day of allergen exposure on day 15, and lungs taken for histological analysis. Groups of mice were also sacrificed on day 15 in the absence of allergen exposure for measurement of platelet FcεRI expression from circulating blood, and (day 15: absence of allergen challenge, day 16, 24 hours post day 15 allergen challenge) for enumeration of CD11c+ cells in lung homogenates.

2. Platelet depletion

In order to produce profound, yet temporary, platelet depletion for the selective period of allergen sensitization, groups of mice were administered intra-muscularly (*i.m.* 50μL into a hindlimb) with 25μg/mouse anti-platelet antibody (anti GP1bα, Emfret Technologies) or control IgG (monoclonal 2A3 anti-trinitrophenol, BioXCell) 24h prior to allergen sensitizations on days 1 and 6. Previous studies in our laboratory have demonstrated that mice receiving this antiplatelet antibody do not display pulmonary oedema, haemorrhage, or weight loss beyond 10% body weight.

3. Statistical Analysis

The experimenters were blinded to the identity of experimental groups for the manual quantification of tissue samples/ cells. Data from allergic animal studies are presented as mean \pm standard error of the mean (SEM), and analysed by one-way ANOVA, followed by Bonferroni's multiple comparison post test. A P value of less than 0.05 was considered significant.

For Review Only

180 Results

181 *CD11c⁺ cell and platelet co-localisation occurs in the airways during sensitization to allergen.*

182
183 Platelets, in low numbers, have previously been observed adjacent to mononuclear cells in
184 lungs of mice before allergen exposure.¹⁶ To identify these cells, and to quantify the extent of
185 platelet decoration, lung sections were stained for CD11c using an anti-mouse anti-CD11c
186 antibody (green, represented by white arrow. **Figure 1A**) as a marker for DCs²⁷ and the
187 presence of platelets using an anti-mouse anti-CD41 antibody (red. **Figures 1B-D**). CD11c⁺ cells
188 were found in close proximity to the airway wall of sham (**Figure 1B**) and allergen-sensitized
189 mice on day 15, before allergen exposure (**Figure 1C**). CD11c⁺ cells in tissue sections from
190 allergen-sensitized mice show evidence of positive CD41 staining (**Figure 1C**), furthermore,
191 individual platelets could be distinguished surrounding CD11c⁺ cells (**Figure 1D**). No difference
192 in the number of CD11c⁺ cells was apparent between sham-sensitized and allergen-sensitized
193 mice before allergen exposure (0 hours) on day 15 around the airway walls (**Figure 1E**). 24
194 hours after allergen exposure (on day 16), a significant increase in CD11c⁺ cell localization was
195 observed localized to the airway wall of allergen-sensitized mice (p<0.05, **Figure 1E**).
196 Interestingly, there was also a significant increase in the incidence of platelets co-localized to
197 CD11c⁺ cells around the airway walls in allergen-sensitized mice when compared to sham -
198 sensitized mice *before* allergen exposure (0 hours) on day 15 (sham-sensitized vs OVA-
199 sensitized p<0.05 **Figure 1F**). This co-localization around the airway wall was further increased
200 24hours post allergen exposure on day 16 (sham-sensitized vs OVA-sensitized p<0.01 **Figure**
201 **1F**). Thus, the observation that platelets co-localized with tissue CD11c⁺ cells in response to

allergen sensitization suggests that platelet activation (e.g. migration, intercellular communication) occurs and may therefore be relevant during allergen recognition by APCs.

Induction of temporary thrombocytopenia at time of allergen sensitization.

To evaluate whether platelets are involved in allergen sensitization, we developed a novel protocol to make mice temporarily thrombocytopenic during, but importantly restricted to, the distinct period of allergen sensitization, rather than during allergen exposure as has been previously reported.^{3,10-12,24-26} Platelet depletion using an anti-GP1b α antibody induced a 95% reduction of circulating platelets when administered 24 hours before each *i.p.* administration of OVA (10 μ g/mouse on days 0 and 7). Platelet counts were measured on day -1, day 0 (30 minutes prior to allergen sensitization), day 9, and day 14. (**Figure 2A**). Platelet counts had recovered by day 14, 24 hours before the start of exposure to aerosolized allergen on day 15 (**Figure 2A**). Importantly, temporary platelet depletion did not affect circulating leukocyte counts (**Figure 2A**).

Platelet expression of Fc ϵ RI is increased during allergen sensitization, but is suppressed in the recovered platelet population when temporary thrombocytopenia occurs during the period of allergen sensitization.

Histological analysis revealed the co-localization of CD11c⁺ cells and platelets in lung tissue in allergen-sensitized mice before allergen exposure and our previous data revealed that the accumulation of platelets into the lung after sensitization was dependent on platelet Fc ϵ RI.¹⁶ Furthermore, we have previously reported that the expression of Fc ϵ RI on platelets is increased in mice sensitized to allergen compared to sham-sensitized mice.¹⁶ We therefore examined the effect of platelet depletion at the time of sensitization on the expression of platelet Fc ϵ RI. Using

a gating strategy to identify CD41+ platelet events by flow cytometry (**Figure 2B**) we identified FcεRI on the surface of these cells (**Figure 2C-E**). On day 15, a significant increase in the proportion of platelets expressing FcεRI was observed in allergen-sensitized mice compared to sham-sensitized mice in the absence of allergen exposure ($p<0.01$, **Figure 2F**). This distinct population of platelets expressing FcεRI was significantly suppressed in allergen-sensitized mice depleted of platelets during the sensitization period compared to allergen-sensitized mice administered control IgG antibody ($p<0.01$ **Figure 2F**). Thus, given that only a distinct population of platelets express FcεRI, and murine platelet lifespan is 3-4 days, the process of sensitization to allergen continues to directly affect the manifestation of this platelet population, or bone marrow megakaryocytes sometime after the platelet population temporally associated with the administration of allergen would have been consumed. These data suggest that the sensitization process towards allergen directly affects the phenotype of circulating platelets.

Temporary thrombocytopenia at time of allergen sensitization inhibits pulmonary leukocyte recruitment in response to subsequent allergen exposure.

On days 15, 16, and 17, mice were exposed to aerosolized allergen (OVA). 24 hours post final allergen exposure (day 18), BALs were performed to enumerate pulmonary leukocyte recruitment. Allergen exposure induced significant pulmonary recruitment of inflammatory cells in allergen-sensitized mice compared to sham-sensitized mice (Eosinophils $p<0.001$ **Figure 3A**; macrophages $p<0.05$ **Figure 3B**; neutrophils $p<0.05$ **Figure 3C**; and lymphocytes $p<0.01$ **Figure 3D**). The presence of thrombocytopaenia at the time of allergen sensitization in mice

suppressed leukocyte recruitment compared to allergen-sensitized mice administered with the non-depleting control IgG antibody (Eosinophils $p < 0.01$ **Figure 3A**; macrophages $p < 0.05$ **Figure 3B**). Lymphocyte recruitment was also suppressed, but not to a significant degree (**Figure 3D**), whilst neutrophil recruitment was not altered in platelet depleted animals (**Figure 3C**).

Temporary thrombocytopenia at the time of allergen sensitization, suppresses IgE and IL-4 production.

We next investigated whether platelet depletion during the phase of allergen sensitization might affect the immune phenotype of the mice. IL-4, IL-5 and IFN- γ levels in lavage fluid, and IgE in serum, were measured from samples taken on day 18, 24 hours after the last day of allergen exposure on day 17. Significantly increased levels of IgE, IL-4, and IL-5, but not IFN- γ , were observed in allergen-sensitized mice compared to sham sensitized mice (IgE: $p < 0.05$ **Figure 4A**; IL-4 $p < 0.05$ **Figure 4B**; IL-5 $p < 0.05$ **Figure 4C**). Temporary thrombocytopenia during the period of allergen sensitization resulted in a suppression of IgE production ($p < 0.05$ **Figure 4A**) and IL-4 production ($p < 0.05$ **Figure 4B**). Whilst IL-5 production (**Figure 4C**) and IFN- γ were not affected (**Figure 4D**); these data nonetheless suggest that platelets directly affect the Th2 sensitization process during this period.

Temporary thrombocytopenia during the sensitization process inhibits pulmonary CD11c⁺ cell recruitment.

We have previously reported that in non-inflamed tissue, there are a small number of platelets that reside in lung tissue, and 90% of these are attached to mononuclear cells.¹⁶ Indeed, there is a 29.7% increase in these events in OVA-sensitized mice compared to sham-sensitized mice

as measured by immunohistochemistry of lung tissue sections for CD41 positive events (Sham: 84.1±20.6 platelets/mm² vs OVA: 109.1±31.9 platelets/mm² from ref ¹⁶). Furthermore, this increased accumulation of platelets extravascularly and their association with mononuclear cells was suppressed in mice deficient in functional FcεRI, suggesting that platelet localization to lung-resident mononuclear cells in response to allergen sensitization was dependent on platelet activation via FcεRI.¹⁶ Given that platelet FcεRI expression is significantly decreased in animals made temporarily thrombocytopaenic at the time of allergen sensitization (**Figure 2F**), we assessed whether the increase in CD11c⁺ cell number in the lungs after allergen exposure (**Figure 1F**) was platelet dependent. Lungs were taken from either sham-sensitized or allergen-sensitized mice at either 0 (day 15) or 24 hours (day 16) after allergen exposure, and processed into a single cell suspension to allow quantitative flow cytometry of CD11c⁺ events with the use of a fixed number of fluorescent beads to assist with accurate enumeration (**Figure 5A**). In accordance with immunohistochemistry of mouse lungs, a significant increase in the number of CD11c⁺ cells was observed 24 hours post allergen exposure on day 16 in allergen-sensitized mice compared to sham-sensitized mice (p<0.001, **Figure 5C**), and was a result of secondary allergen exposure, rather than the sensitization process, since no increase in CD11c⁺ cells was observed before allergen exposure (0 hours) on day 15 (**Figure 5B**). This accumulation was reduced in allergen-sensitized mice rendered temporarily thrombocytopaenic during the period of allergen sensitization (p<0.05, **Figure 5C**), suggesting that allergen-exposed platelets are necessary for pulmonary CD11c⁺ cell recruitment.

296

297 **Discussion**

298 We have shown for the first time that platelets are causal in the initial processing of allergen *in*
 299 *vivo*. The temporary depletion of platelets during the initial sensitization period suppressed
 300 inflammatory cell recruitment upon subsequent allergen exposure (aerosol allergen challenge,
 301 **Figure 3**) and this correlated with an inhibition of IL-4 synthesis and IgE production (**Figure 4**).
 302 We have previously reported that allergen can induce platelet motility and migration into lungs
 303 after allergen exposure,¹⁶ whilst others have also reported activation of platelets via IgE-
 304 dependent mechanisms.¹⁸ As we report here, one consequence of platelet accumulation into
 305 tissue in response to allergen is their co-localisation with CD11c⁺ cells, a phenomenon that
 306 occurs before subsequent allergen exposure (**Figure 1**). Given the reduction in the Th2
 307 response, we conclude that the actions of platelets during allergen-sensitization are probably
 308 delivered via an IgE-dependent FcεRI process to interact with lung APCs, for example CD11c⁺
 309 DCs to process allergen signalling with T-cells in bronchial lymph nodes, although the data in
 310 this manuscript are restricted to CD11c⁺ cells, rather than specifically DCs that express the
 311 CD11c antigen. We note that in contrast to other studies,²⁴ we do not observe effects of
 312 platelet depletion on IgE and Th2 cytokine production after allergen exposure, either acutely (3
 313 days of allergen exposure) or chronically (8 weeks of allergen exposure), and the effects of
 314 platelets on Th2 cytokine production are constrained temporally to the time of allergen
 315 sensitization.^{3,13,25}

316 The phenomenon that allergen-exposed platelets are necessary for pulmonary CD11c⁺ cell
317 recruitment after secondary allergen exposure was unlikely to be due to an inability of the
318 restored platelet population to efficiently recruit CD11c⁺ cells from the circulation, as our
319 previous studies have revealed platelets from non allergen-sensitized mice retain their ability to
320 recruit leukocytes.³ Furthermore, neutrophil recruitment to the lungs was not influenced by
321 platelet depletion at the time of allergen sensitization, even though it is well understood that
322 pulmonary neutrophil recruitment is dependent on intravascular interactions with platelets.^{10,}
323 ²⁶

324 It remains to be understood why professional APCs such as DCs might require platelet
325 interactions for a more efficient processing of allergen *in vivo*. DCs also express FcεRI in
326 different conformations with cross-linked IgE, yet it has been reported that IgE-mediated
327 activation of DCs in allergic Th-2 type inflammation might be immune-regulatory rather than
328 pro-inflammatory.^{27,28} Clearly, IgE/FcεRI-mediated antigen presentation by DCs is considered to
329 be a critical event in the priming of Th2-type cells,²⁹ but recent evidence suggests that this
330 response is dependent on low antigen concentrations, rather than receptor signalling *per se*.³⁰ T
331 cell activities induced after IgE/FcεRI dependent processes by DCs have been reported not to
332 develop as efficiently into Th2-type effector cells than those generated via an IgE/FcεRI
333 independent process.³¹ How DCs process antigen and elicit a Th-2 type response is complex,
334 and might therefore involve other cell types, including platelets that have been implicated in
335 the innate response and express FcεRI, CD23, and other Immunoglobulin receptors, as well as
336 toll-like receptors (TLRs).² Indeed, it has been reported that platelet communication with APCs
337 is regulated by CD40/CD40L interactions, and this might be an important component of DC

communication with T cells in the draining lymph nodes after platelet activation by allergen.^{32,33} Currently, our understanding as to why APCs might require platelets to effect an immune response is incomplete, but perhaps platelet activation by allergen or pathogens via IgE-dependent processes is a more direct, innate response, with less regulation and control. Two recent studies report the ability of platelets to modulate the immune response in mouse models of eosinophilic and allergic airway inflammation.^{34,35} The transfer of human platelets restored papain-induced airway eosinophilia on an IL-33 deficient background in mice, supporting a hypothesis that platelets are important in the IL-33 dependent provocation of Th2 immune cells.³⁴ Furthermore, the platelet derived Wnt antagonist Dickkopf-1 was reported to be important for Th2 cell cytokine production in a house dust mite (HDM) model of asthma.³⁵ The pathways by which platelets might affect sensitization to allergens in patients with asthma has not yet been described, but it is of interest that platelets from patients allergic to *Dermatophagoides pteronyssinus* have been shown to be activated directly by the specific allergen, and not activated by other known allergens to which the allergic blood donor was not sensitized to.^{16,18} Thus, clinical data is suggestive of a interaction between platelets and the sensitization process.

It is not understood how induced expression of FcεRI occurs on circulating platelets (**Figure 2**). The percentage of platelets that express these receptors remains a small subset of the whole platelet population, which might suggest that megakaryocytes residing in the bone marrow are capable of differentiation to ultimately determine the phenotype of subsets of platelets. It is

interesting to note however, that this increase in platelet FcεRI expression is suppressed in mice subjected to the temporary thrombocytopaenia at the time of allergen sensitization (**Figure 2**). The lifespan of murine platelets is between 3-4 days, yet the recording was taken when the platelet population had fully recovered, suggesting that there is an establishment of immune memory in a subset of megakaryocytes to continue producing a population of platelets with increased expression of FcεRI.

To conclude, the initial processing of antigen in naive animals requires the involvement of platelets, and leads to an increased production of allergen responsive platelets perhaps transmitted from megakaryocytes during thrombopoiesis, and supports earlier clinical data reporting the ability of platelets to become activated by specific allergens to which individual patients with allergy become sensitized to.^{16,18,36} These platelets respond by recruiting to organs that are exposed to the external environment and migrate to prime resident APCs for an efficient antigen-dependent immune response after subsequent exposure to allergen.

Figure Legends

Figure 1. Association of platelets with CD11c⁺ cells in lungs of allergen-sensitized mice. Lungs from either sham-sensitized or allergen sensitized (OVA) mice were collected at 0, 24, or 48 hours after exposure to aerosolised OVA from day 15. Lung sections were stained for the presence of CD41⁺ (platelets, red fluorescence) and CD11c⁺ cells (green fluorescence): Representation of CD11c⁺ cell (white arrow) (A); platelets and CD11c⁺ cells localised to airways in sham sensitized (B) and allergen-sensitized mice (C). Arrows indicate presence of CD11c⁺ cells. A 'zoomed' image (using Microsoft Office) represented in the dotted oblong (C) shows individual platelets (red) attached to the CD11c⁺ cell (D). Images photographed using a x40 objective under a Zeiss Axiovert microscope. Images A-D were enhanced to improve brightness/contrast using Microsoft Powerpoint. The presence of CD11c⁺ cells was quantified around airway walls (E). The percentage of CD11c⁺ cells co-localized with CD41⁺ platelets was determined within the airway walls (F). n=4 per group. **P< 0.01, *P< 0.05 compared to sham control group.

Figure 2. Induction of temporary thrombocytopaenia during the period of allergen sensitization and effects on platelet FcεRI expression. Mice were administered either a control IgG antibody, or platelet-depleting anti-GPIIb/IIIa antibody on days -1 and 6 of the sensitization procedure to deplete circulating platelets within the time frame of allergen sensitization (OVA) on days 0 and 7 (A). Tail vein bleeds were conducted on days -1, 0, 9, and 14 to enumerate

406 circulating platelet and leukocyte numbers (A). Representative histograms show CD41+
 407 platelets from lysed whole blood (B), FcεRI expression in sham-sensitized (C), OVA-sensitized +
 408 IgG (D), and OVA-sensitized + anti-GPIbα mice (E). Expression recorded as % of cells expressing
 409 antigen on day 15 in the absence of secondary allergen exposure (F). n=4-5 per group. Data:
 410 means ±SEM. **P< 0.01 versus sham-sensitised control group, ##P<0.01 versus OVA-sensitised
 411 IgG group.

412

413 **Figure 3. Effects of temporary thrombocytopaenia during the period of allergen sensitization**
 414 **on the inflammatory response after subsequent allergen exposure.** Mice were administered
 415 either a control IgG antibody, or platelet-depleting anti-GPIbα antibody on days -1 and 6 of the
 416 sensitization procedure to deplete circulating platelets within the time frame of allergen
 417 sensitization (OVA) on days 0 and 7. On days 15, 16, and 17, mice were subsequently exposed
 418 to aerosolized allergen (OVA) for 30 mins/day before BAL was performed on day 18 and
 419 enumerated for eosinophils (B), macrophages (C), neutrophils (D), and lymphocytes (E). n=8-12
 420 per group. Data: means± SEM. *P< 0.05, **P< 0.01, ***P< 0.001 versus sham-sensitised control
 421 group. #P< 0.05, ##P< 0.01, versus OVA-sensitised group (OVA+ IgG).

422

423 **Figure 4. Effects of temporary thrombocytopaenia during the period of allergen sensitization**
 424 **on the cytokine expression and IgE titre after subsequent allergen exposure.** Mice were
 425 administered either a control IgG antibody, or platelet-depleting anti-GPIbα antibody on days -1
 426 and 6 of the sensitization procedure to deplete circulating platelets within the time frame of

allergen sensitization (OVA) on days 0 and 7. On days 15, 16, and 17, mice were subsequently exposed to aerosolized allergen (OVA) for 30 mins/day before BAL and blood was harvested on day 18 for plasma IgE analysis (A), and BAL enumerated for IL-4 (B), IL-5 (C), and IFN γ (D) titres. n=6-8 per group. Data: means \pm SEM. *P< 0.05 versus sham-sensitised control group. #P< 0.05 versus OVA-sensitised group (OVA+ IgG).

Figure 5. Effect of platelets and allergen on DC recruitment. Allergen-sensitized mice had previously been administered either control IgG or platelet-depleting anti-GPIb α antibody during the allergen sensitization period, and lungs were harvested at 0 (day 15) and 24 hours after (day 16) exposure to aerosolized allergen (OVA) for histology or flow cytometry. Lungs were processed into a single cell suspension and CD11c⁺ cells were quantified via flow cytometry using fluorospheres to obtain an accurate count (A) at 0 hours (B), 24 hours (C) post allergen challenge. n=3-9. Data: means \pm SEM. ***P< 0.001 versus sham-sensitized group; and #P< 0.05 versus OVA-sensitized IgG group

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Figure 1

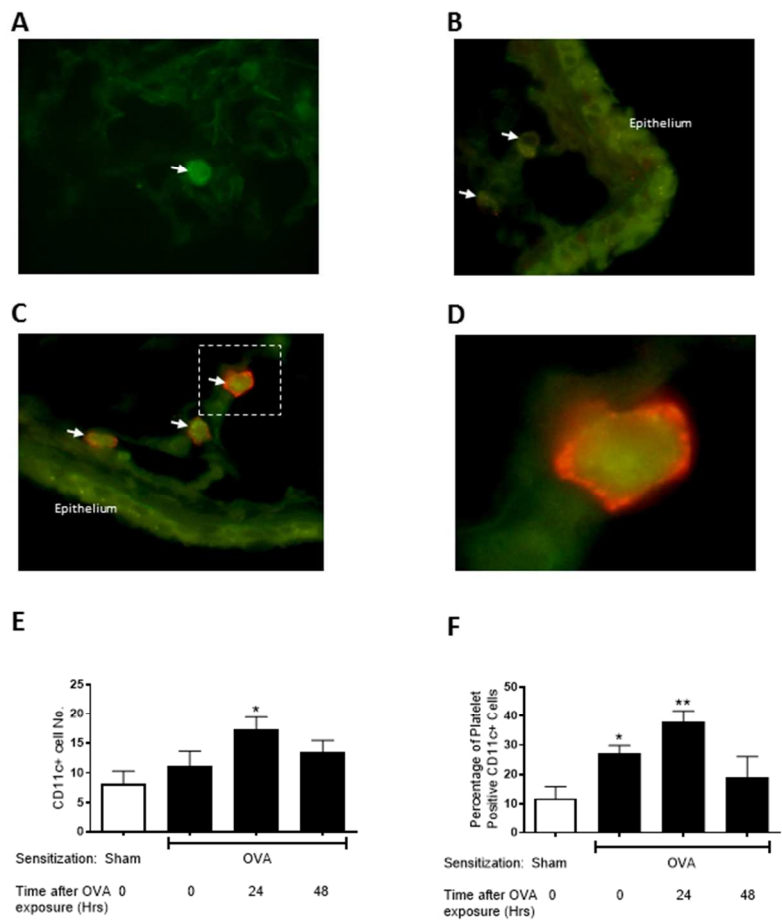


Figure 1

190x254mm (96 x 96 DPI)

Figure 2

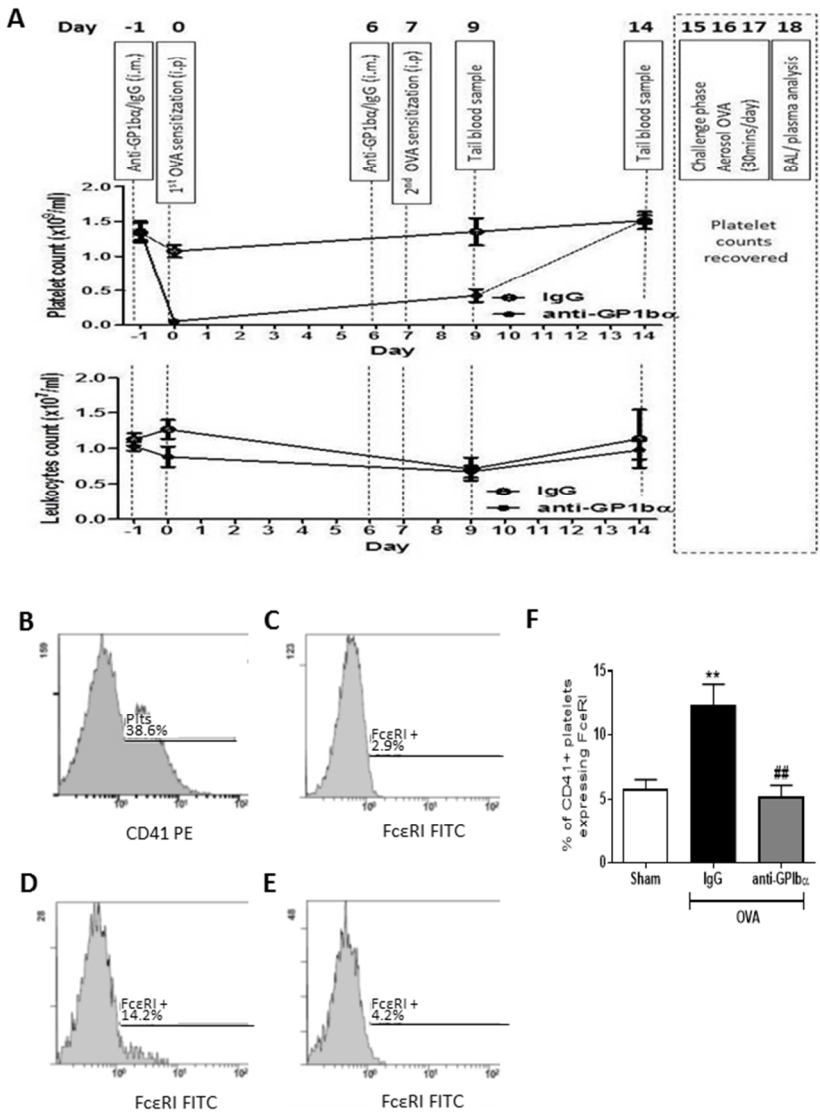


Figure 2

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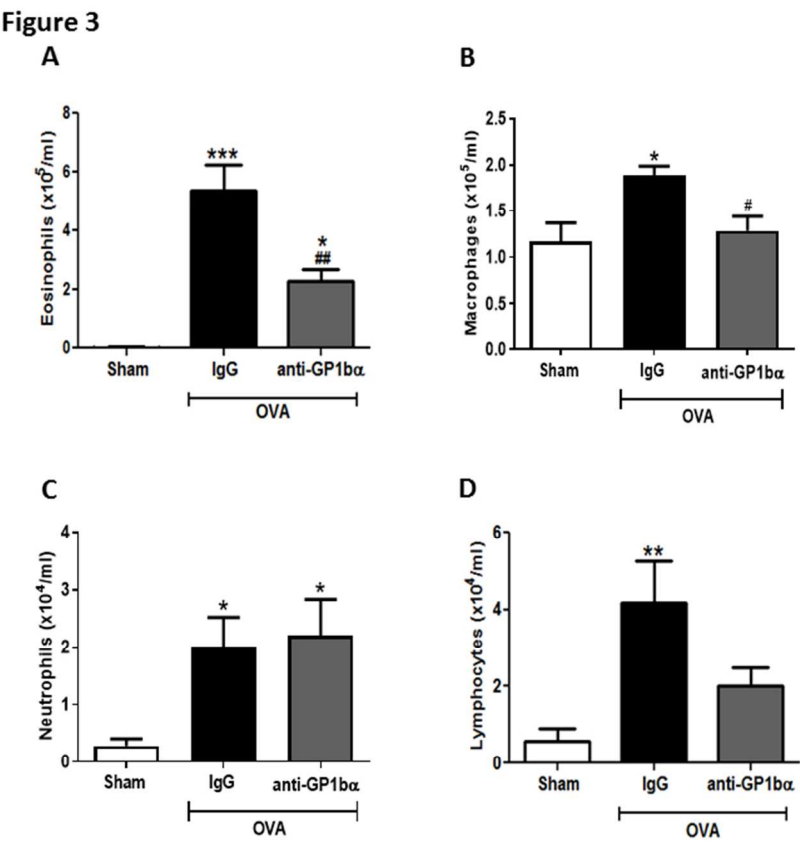


Figure 3
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Figure 4

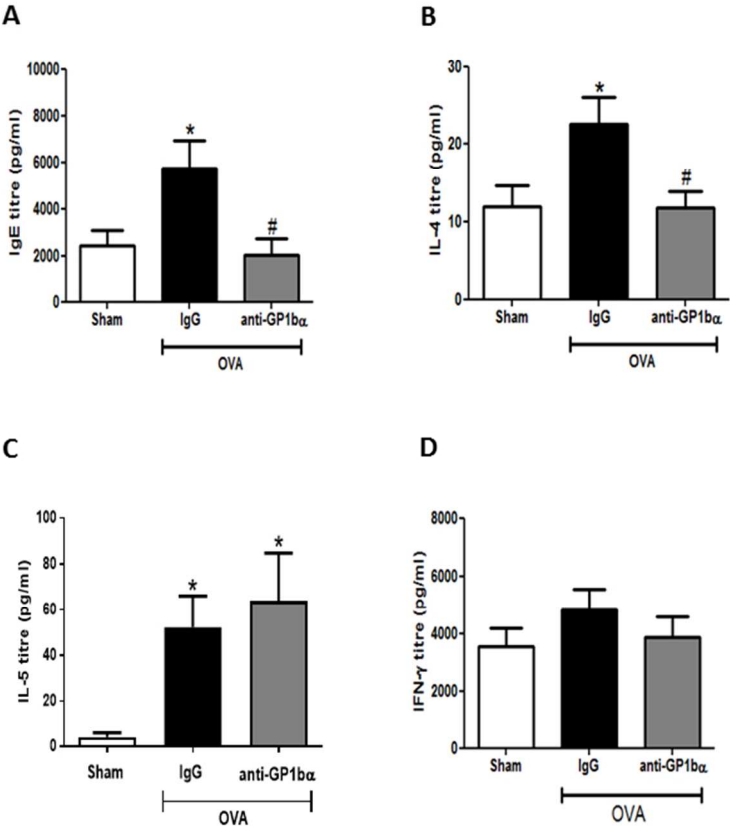


Figure 4

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Figure 5

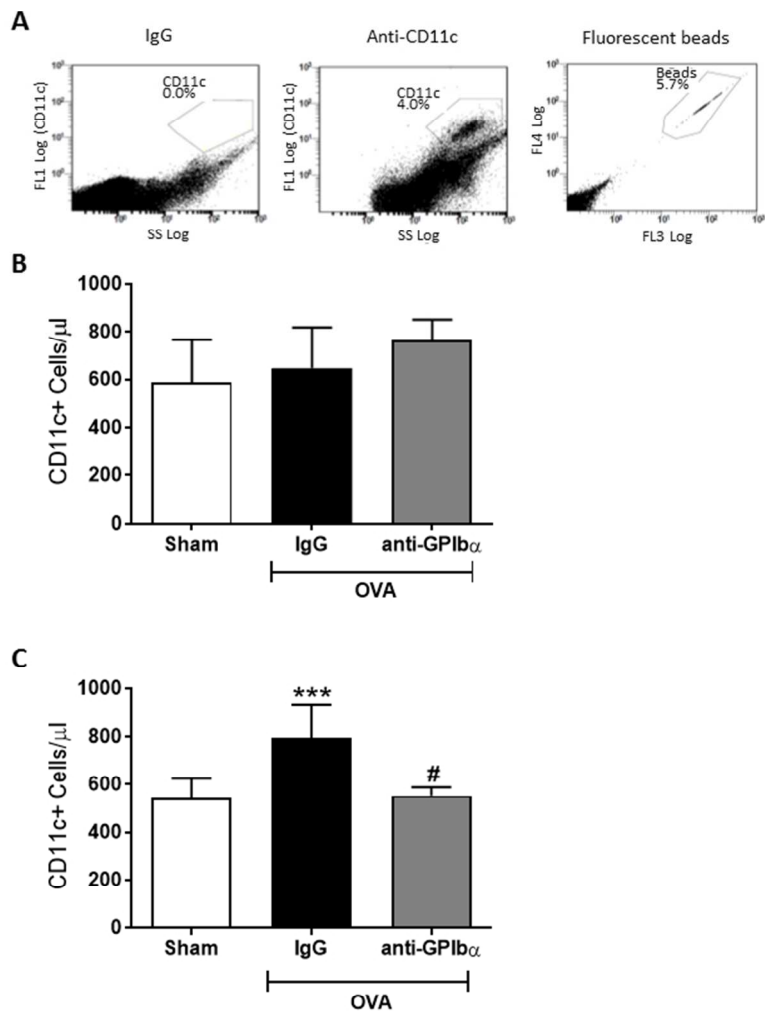


Figure 5

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ONLINE SUPPLEMENTARY MATERIAL

Platelets play a central role in sensitization to allergen.

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A. Blood microsampling

For blood microsampling to enumerate circulating platelet and leukocyte numbers, mice were placed in a heated chamber (37 °C) for 10 minutes before a 1-2 mm cut was made with a scalpel blade across the lateral tail vein. Using a micropipette, 2 µl of blood was diluted 1:100 into stromatol solution for platelet counts, a further 2 µl diluted 1:50 in Turk's solution for leukocyte counts (both using a Neubauer improved haemocytometer). Manual counts were made using a Zeiss Axiovert microscope.

B. Immunohistochemistry of lungs.

At 0h, 24h, and 48h, post one day of allergen challenge on day 15, lungs were recovered from terminally anaesthetized mice. Lungs were inflated with a tracheal cannula with 4% PFA and then immersed to fix in 4% PFA for 1 hour. The lungs were then incubated in 10% sucrose solution (in PBS) overnight at 4°C. The sucrose solution was then replaced with 25% OCT (in PBS) for 1 hour, and then with 100% OCT for 1 hour. Lungs were then stored at -80°C, after which they were sectioned (6µm) with a Cryostat and placed on poly-L-lysine coated microscope slides. Tissue sections were stained with primary antibodies to detect CD11c⁺ cells (hamster anti-mouse CD11c Integrin α x chain, HL3, BD Biosciences) and

platelets (Goat polyclonal IgG Integrin α IIb. β 3, Santa Cruz Biotech), for 2 hours. Slides containing lung sections were then washed and incubated with secondary fluorescent antibodies: 1. Donkey anti-goat IgG-Cy3 for 1 hour, before PBS wash followed by 2. Goat-anti Armenian hamster IgG-FITC for 1 hour. Finally, slides were cover slipped using a photo bleaching protecting mountant and stored in the dark at 4°C until required for microscopic analysis.

C. Microscopic analysis and Lung morphometry

Lung section slides were analysed under a fluorescent microscope (Zeiss Axioskop) using a x100 objective, where CD11c+ cell localisation was measured around airway walls to a depth of 50 μ m beneath the sub-epithelial basement membrane, in a five field area. CD11c+ cells were investigated for platelet co-localisation (CD41+ events) and the extent of those with positive results quantified per airway using an image analysis programme (Scion Image; National Institutes of Health, Bethesda, MD).

D. Flow cytometric analysis of CD11c+ cells and platelets

All experiments were analysed on a flow cytometer (FC500 series Beckman Coulter) as previously described.^{10,16} Single cell suspensions isolated from lung digestion at 0 and 24 hours after allergen challenge on day 15 were incubated with the DC marker, anti-CD11c-fluorescein mAb, in a saturating concentration for 30 minutes at room temperature away from direct light. For quantification of CD11c+ cells, samples were also treated with Beckman coulter Flow CountTM fluorosphere beads (Beckman Coulter). Specific gating parameters were set up to calibrate for the assayed concentration of the fluorospheres to

allow absolute quantification of anti-CD11c-fluorescein positive events. For analysis of Platelet FcεRI expression, citrated blood was obtained via cardiac puncture on other groups of mice on day 15 in the absence allergen challenge (0 hours). Blood was incubated with platelet specific anti-CD41-phycoerythrin mAb and anti-FcεRI-fluorescein mAb in saturating concentrations for 30 minutes at room temperature and platelets were gated on size and FcεRI positive events were then measured on CD41 positive cells.

E. Quantification of IgE, IL-4, IL-5, and IFN-γ

Bronchoalveolar lavage samples taken at day 18 were centrifuged at 300 G for 5 minutes and supernatants harvested for quantification of *IL-4*, *IL-5*, and *IFN-γ* using specific ELISAs as per the manufacturer's instructions (DuoSet, R&D systems). Total IgE was measured in plasma samples prepared from citrated blood harvested via cardiac puncture at day 18. A murine specific IgE ELISA was used as per the manufacturer's instructions (BD Pharmingen. Primary capture antibody: rat anti-mouse IgE clone R35-72).

Platelets play a central role in sensitization to allergen.

Running Title: Platelets and allergen sensitization.

Keywords: Platelets, allergen sensitization, FcεRI, IgE, IL-4, CD11c, dendritic cells, leukocyte recruitment, asthma

Word count: Abstract: 252, Body of text: 3300, number of figures: 5

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Abstract

Background: Platelet activation occurs in patients with allergic inflammation, and platelets can be activated directly by allergen via an IgE-dependent process. Platelets have been shown to activate antigen-presenting cells (APCs) such as CD11c⁺ dendritic cells (DCs) *in vitro*. Whilst CD11c⁺ DCs are requisite for allergen sensitization, the role of platelets in this process is unknown.

Objectives: We investigated whether platelets were necessary for allergen sensitization.

Methods: Balb/c mice sensitised to ovalbumin (OVA), were exposed to subsequent aerosolized allergen (OVA challenge). We analysed lung CD11c⁺ cell activation, co-localization with platelets, and some other indices of inflammation. The role of platelets at the time of allergen sensitization was assessed through platelet depletion experiments restricted to the period of sensitization.

Results: Platelets co-localized with airway CD11c⁺ cells, and this association increased after allergen sensitization, and after subsequent allergen exposure. Temporary platelet depletion (>95%) at the time of allergen sensitization led to a suppression of IgE and IL-4 synthesis, and a decrease in the pulmonary recruitment of eosinophils, macrophages, and lymphocytes after subsequent allergen exposure. Furthermore, in mice previously depleted of platelets at the time of sensitization, the recovered platelet population were shown to have reduced expression of FcεRI. Pulmonary CD11c⁺ cell recruitment was suppressed in these mice after allergen challenge, suggesting the migration of CD11c⁺ cells *in vivo* may be dependent on direct platelet recognition of allergen.

Conclusion: Platelets are necessary for efficient host sensitization to allergen. This propagates the subsequent inflammatory response during secondary allergen exposure, and increases platelet association with airway CD11c⁺ cells.

Keywords: Platelets, allergen sensitization, FcεRI, IgE, IL-4, CD11c, dendritic cells, leukocyte recruitment, asthma

List of abbreviations used

APC, antigen presenting cell

BALF, broncho-alveolar lavage fluid

DCs, Dendritic cells

OVA, Ovalbumin

Introduction

Platelet activation in allergic diseases such as asthma, has been reported extensively over the last 30 years, with evidence of changes in platelet volume, presence of platelet micro-particles, release of platelet-specific inflammatory mediators, and an increased incidence of circulating platelet-leukocyte (including eosinophil) complexes, both in patients undergoing spontaneous asthma attacks or after allergen challenge.¹⁻⁴ Furthermore, platelet activation correlates strongly to changes in lung function.⁴⁻⁹ Various *in vivo* studies confirm that the activation of platelets during allergy and asthma plays a central role in the inflammatory response.^{3,10-13} Of significance, pulmonary platelet recruitment and accumulation occurs in the lungs, initially via an IgE-dependent process whereby platelets migrate and enter the extravascular space.^{7,14-17} The significance and temporal nature of allergen-induced activation of platelets, via FcεRI, has not been established, yet of pertinence to allergy, platelets from patients allergic to *Dermatophagoides pteronyssinus* have been shown to be activated directly by the specific allergen, or by synthetic peptides derived from the allergen, through an IgE dependent process.^{16,18} Combined, these studies argue for a causal role of platelet activation in asthma and allergy, and that such activation is not as a consequence of the inflammatory milieu *per se*. Furthermore, it has been shown that platelet-derived 5-HT is also important for dendritic cell (DC) activation during allergic airways inflammation, further supporting a role for platelets, and suggesting that the activation and migration of platelets induced by allergen might be of significance or causal to the sensitization process.¹⁹ Myeloid DCs (mDCs) are thought to be involved very early in the development of asthma by inducing sensitization to inhaled antigen, and increasingly, mDCs are viewed as essential pro-

100 inflammatory cells that induce effector cytokine production in primed Th2 cells during airway
101 inflammation.²⁰⁻²³ It is therefore of interest that platelet CD40L has recently been reported to
102 be involved in the promotion of allergic airways inflammation by polarizing Th2 responses *after*
103 allergen exposure.²⁴ We have previously demonstrated a presence of platelets extra-vascularly
104 in the lungs of mice before subsequent allergen exposure in allergen-sensitized mice, but the
105 vast majority of these platelets were attached to a resident undefined mononuclear cell
106 population, and the relevance of this occurrence has yet to be established.¹⁶ We hypothesized
107 that these platelets are a distinct population that had migrated to the lungs as a result of
108 exposure to allergen (ip injection of ovalbumin, OVA), and that a population of platelets might
109 therefore be involved in initial sensitization to allergen. Temporally, the occurrence was clearly
110 unrelated to subsequent aerosolized exposures to allergen (OVA), which drives platelet
111 migration into the lungs along with recruited leukocytes.¹⁶ We report here that CD11c⁺ cells (as
112 a marker for DCs) are decorated with platelets during allergen sensitization, and investigate the
113 importance of these platelets during the process of allergen sensitization and their interactions
114 with CD11c⁺ cells, before and during subsequent exposure to aerosolized allergen. These
115 studies provide a possible role for the specific activation of platelets by allergen from patients
116 with asthma, and the occurrence of platelets in the lungs of patients with asthma.

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119

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Materials and Methods

Readers are directed to the online supplementary material document for methodology related to: Blood microsampling, Immunohistochemistry of lungs, Microscopic analysis and lung morphometry, Flow cytometric analysis of CD11c+ cells and platelets, and: Quantification of IgE, IL-4, IL-5, and IFN- γ .

1. Sensitisation of mice to ovalbumin and bronchoalveolar lavage (BAL).

To understand the importance of platelets during allergen sensitization, and subsequent effects on the inflammatory response after secondary allergen exposure via an aerosol challenge, a murine model of allergic sensitization and lung inflammation was used where the sensitization process can be delineated and confined by a defined route of administration (intraperitoneal route) compared to the more complex situation arising from continual exposure to allergen via the airways. All studies were carried out under the Animals (Scientific Procedures) Act of 1986, and amendments 2012 (United Kingdom) with local ethical approval from King's College, London. Studies and reporting have been undertaken under the ARRIVE guidelines. Female Balb/c mice (20-25g; Harlan UK), housed under standard husbandry conditions of 12 hour light/dark cycle, 21-23°C, in groups of 4-6 animals per cage with access to pelleted chow diet and water *ad libitum*, were randomly assigned to different treatment groups. Mice were sensitised with chicken egg ovalbumin (OVA, grade V, Sigma-Aldrich; 10 μ g/0.4ml, absorbed in a saturated solution of an adjuvant: aluminium hydroxide, 0.1M, Sanofi Winthrop), or received

142 adjuvant only (sham-sensitized mice), intraperitoneally (i.p.) on days 0 and 7. On days 15, 16,
 143 and 17, mice were subsequently nebulised with OVA (10mg/ml) for 30 minutes, 24 hours post
 144 final allergen challenge (day 18). BAL fluid was processed for total and differential cell counts,
 145 and the enumeration of cytokines, whilst blood was processed for measurement of total IgE
 146 content as previously described.^{10,25} Some groups of mice were sacrificed 0, 24, and 48h after
 147 one day of allergen exposure on day 15, and lungs taken for histological analysis. Groups of
 148 mice were also sacrificed on day 15 in the absence of allergen exposure for measurement of
 149 platelet FcεRI expression from circulating blood, and (day 15: absence of allergen challenge, day
 150 16, 24 hours post day 15 allergen challenge) for enumeration of CD11c+ cells in lung
 151 homogenates.

152

153 2. Platelet depletion

154 In order to produce profound, yet temporary, platelet depletion for the selective period of
 155 allergen sensitization, groups of mice were administered intra-muscularly (*i.m.* 50μL into a
 156 hindlimb) with 25μg/mouse anti-platelet antibody (anti GP1bα, Emfret Technologies) or control
 157 IgG (monoclonal 2A3 anti-trinitrophenol, BioXCell) 24h prior to allergen sensitizations on days -
 158 1 and 6. Previous studies in our laboratory have demonstrated that mice receiving this
 159 antiplatelet antibody do not display pulmonary oedema, haemorrhage, or weight loss beyond
 160 10% body weight.

161

162 3. Statistical Analysis

The experimenters were blinded to the identity of experimental groups for the manual quantification of tissue samples/ cells. Data from allergic animal studies are presented as mean \pm standard error of the mean (SEM), and analysed by one-way ANOVA, followed by Bonferroni's multiple comparison post test. A P value of less than 0.05 was considered significant.

For Review Only

180 Results

181 *CD11c⁺ cell and platelet co-localisation occurs in the airways during sensitization to allergen.*

182
 183 Platelets, in low numbers, have previously been observed adjacent to mononuclear cells in
 184 lungs of mice before allergen exposure.¹⁶ To identify these cells, and to quantify the extent of
 185 platelet decoration, lung sections were stained for CD11c using an anti-mouse anti-CD11c
 186 antibody (green, represented by white arrow. **Figure 1A**) as a marker for DCs²⁷ and the
 187 presence of platelets using an anti-mouse anti-CD41 antibody (red. **Figures 1B-D**). CD11c⁺ cells
 188 were found in close proximity to the airway wall of sham (**Figure 1B**) and allergen-sensitized
 189 mice on day 15, before allergen exposure (**Figure 1C**). CD11c⁺ cells in tissue sections from
 190 allergen-sensitized mice show evidence of positive CD41 staining (**Figure 1C**), furthermore,
 191 individual platelets could be distinguished surrounding CD11c⁺ cells (**Figure 1D**). No difference
 192 in the number of CD11c⁺ cells was apparent between sham-sensitized and allergen-sensitized
 193 mice before allergen exposure (0 hours) on day 15 around the airway walls (**Figure 1E**). 24
 194 hours after allergen exposure (on day 16), a significant increase in CD11c⁺ cell localization was
 195 observed localized to the airway wall of allergen-sensitized mice (p<0.05, **Figure 1E**).
 196 Interestingly, there was also a significant increase in the incidence of platelets co-localized to
 197 CD11c⁺ cells around the airway walls in allergen-sensitized mice when compared to sham -
 198 sensitized mice *before* allergen exposure (0 hours) on day 15 (sham-sensitized vs OVA-
 199 sensitized p<0.05 **Figure 1F**). This co-localization around the airway wall was further increased
 200 24hours post allergen exposure on day 16 (sham-sensitized vs OVA-sensitized p<0.01 **Figure**
 201 **1F**). Thus, the observation that platelets co-localized with tissue CD11c⁺ cells in response to

allergen sensitization suggests that platelet activation (e.g. migration, intercellular communication) occurs and may therefore be relevant during allergen recognition by APCs.

Induction of temporary thrombocytopenia at time of allergen sensitization.

To evaluate whether platelets are involved in allergen sensitization, we developed a novel protocol to make mice temporarily thrombocytopenic during, but importantly restricted to, the distinct period of allergen sensitization, rather than during allergen exposure as has been previously reported.^{3,10-12,24-26} Platelet depletion using an anti-GP1b α antibody induced a 95% reduction of circulating platelets when administered 24 hours before each *i.p.* administration of OVA (10 μ g/mouse on days 0 and 7). Platelet counts were measured on day -1, day 0 (30 minutes prior to allergen sensitization), day 9, and day 14. (Figure 2A). Platelet counts had recovered by day 14, 24 hours before the start of exposure to aerosolized allergen on day 15 (Figure 2A). Importantly, temporary platelet depletion did not affect circulating leukocyte counts (Figure 2A).

Platelet expression of Fc ϵ RI is increased during allergen sensitization, but is suppressed in the recovered platelet population when temporary thrombocytopenia occurs during the period of allergen sensitization.

Histological analysis revealed the co-localization of CD11c⁺ cells and platelets in lung tissue in allergen-sensitized mice before allergen exposure and our previous data revealed that the accumulation of platelets into the lung after sensitization was dependent on platelet Fc ϵ RI.¹⁶ Furthermore, we have previously reported that the expression of Fc ϵ RI on platelets is increased in mice sensitized to allergen compared to sham-sensitized mice.¹⁶ We therefore examined the effect of platelet depletion at the time of sensitization on the expression of platelet Fc ϵ RI. Using

a gating strategy to identify CD41+ platelet events by flow cytometry (**Figure 2B**) we identified FcεRI on the surface of these cells (**Figure 2C-E**). On day 15, a significant increase in the proportion of platelets expressing FcεRI was observed in allergen-sensitized mice compared to sham-sensitized mice in the absence of allergen exposure ($p < 0.01$, **Figure 2F**). This distinct population of platelets expressing FcεRI was significantly suppressed in allergen-sensitized mice depleted of platelets during the sensitization period compared to allergen-sensitized mice administered control IgG antibody ($p < 0.01$ **Figure 2F**). Thus, given that only a distinct population of platelets express FcεRI, and murine platelet lifespan is 3-4 days, the process of sensitization to allergen continues to directly affect the manifestation of this platelet population, or bone marrow megakaryocytes sometime after the platelet population temporally associated with the administration of allergen would have been consumed. These data suggest that the sensitization process towards allergen directly affects the phenotype of circulating platelets.

Temporary thrombocytopenia at time of allergen sensitization inhibits pulmonary leukocyte recruitment in response to subsequent allergen exposure.

On days 15, 16, and 17, mice were exposed to aerosolized allergen (OVA). 24 hours post final allergen exposure (day 18), BALs were performed to enumerate pulmonary leukocyte recruitment. Allergen exposure induced significant pulmonary recruitment of inflammatory cells in allergen-sensitized mice compared to sham-sensitized mice (Eosinophils $p < 0.001$ **Figure 3A**; macrophages $p < 0.05$ **Figure 3B**; neutrophils $p < 0.05$ **Figure 3C**; and lymphocytes $p < 0.01$ **Figure 3D**). The presence of thrombocytopaenia at the time of allergen sensitization in mice

suppressed leukocyte recruitment compared to allergen-sensitized mice administered with the non-depleting control IgG antibody (Eosinophils $p < 0.01$ **Figure 3A**; macrophages $p < 0.05$ **Figure 3B**). Lymphocyte recruitment was also suppressed, but not to a significant degree (**Figure 3D**), whilst neutrophil recruitment was not altered in platelet depleted animals (**Figure 3C**).

Temporary thrombocytopenia at the time of allergen sensitization, suppresses IgE and IL-4 production.

We next investigated whether platelet depletion during the phase of allergen sensitization might affect the immune phenotype of the mice. IL-4, IL-5 and IFN- γ levels in lavage fluid, and IgE in serum, were measured from samples taken on day 18, 24 hours after the last day of allergen exposure on day 17. Significantly increased levels of IgE, IL-4, and IL-5, but not IFN- γ , were observed in allergen-sensitized mice compared to sham sensitized mice (IgE: $p < 0.05$ **Figure 4A**; IL-4 $p < 0.05$ **Figure 4B**; IL-5 $p < 0.05$ **Figure 4C**). Temporary thrombocytopenia during the period of allergen sensitization resulted in a suppression of IgE production ($p < 0.05$ **Figure 4A**) and IL-4 production ($p < 0.05$ **Figure 4B**). Whilst IL-5 production (**Figure 4C**) and IFN- γ were not affected (**Figure 4D**); these data nonetheless suggest that platelets directly affect the Th2 sensitization process during this period.

Temporary thrombocytopenia during the sensitization process inhibits pulmonary CD11c⁺ cell recruitment.

We have previously reported that in non-inflamed tissue, there are a small number of platelets that reside in lung tissue, and 90% of these are attached to mononuclear cells.¹⁶ Indeed, there is a 29.7% increase in these events in OVA-sensitized mice compared to sham-sensitized mice

275 as measured by immunohistochemistry of lung tissue sections for CD41 positive events (Sham:
 276 84.1 ± 20.6 platelets/ mm^2 vs OVA: 109.1 ± 31.9 platelets/ mm^2 from ref ¹⁶). Furthermore, this
 277 increased accumulation of platelets extravascularly and their association with mononuclear
 278 cells was suppressed in mice deficient in functional Fc ϵ RI, suggesting that platelet localization to
 279 lung-resident mononuclear cells in response to allergen sensitization was dependent on platelet
 280 activation via Fc ϵ RI.¹⁶ Given that platelet Fc ϵ RI expression is significantly decreased in animals
 281 made temporarily thrombocytopaenic at the time of allergen sensitization (**Figure 2F**), we
 282 assessed whether the increase in CD11c⁺ cell number in the lungs after allergen exposure
 283 (**Figure 1F**) was platelet dependent. Lungs were taken from either sham-sensitized or allergen-
 284 sensitized mice at either 0 (day 15) or 24 hours (day 16) after allergen exposure, and processed
 285 into a single cell suspension to allow quantitative flow cytometry of CD11c⁺ events with the use
 286 of a fixed number of fluorescent beads to assist with accurate enumeration (**Figure 5A**). In
 287 accordance with immunohistochemistry of mouse lungs, a significant increase in the number of
 288 CD11c⁺ cells was observed 24 hours post allergen exposure on day 16 in allergen-sensitized
 289 mice compared to sham-sensitized mice ($p < 0.001$, **Figure 5C**), and was a result of secondary
 290 allergen exposure, rather than the sensitization process, since no increase in CD11c⁺ cells was
 291 observed before allergen exposure (0 hours) on day 15 (**Figure 5B**). This accumulation was
 292 reduced in allergen-sensitized mice rendered temporarily thrombocytopaenic during the period
 293 of allergen sensitization ($p < 0.05$, **Figure 5C**), suggesting that allergen-exposed platelets are
 294 necessary for pulmonary CD11c⁺ cell recruitment.

295

296

297 **Discussion**

298 We have shown for the first time that platelets are causal in the initial processing of allergen *in*
299 *vivo*. The temporary depletion of platelets during the initial sensitization period suppressed
300 inflammatory cell recruitment upon subsequent allergen exposure (aerosol allergen challenge,
301 [Figure 3](#)) and this correlated with an inhibition of IL-4 synthesis and IgE production ([Figure 4](#)).
302 We have previously reported that allergen can induce platelet motility and migration into lungs
303 after allergen exposure,¹⁶ whilst others have also reported activation of platelets via IgE-
304 dependent mechanisms.¹⁸ As we report here, one consequence of platelet accumulation into
305 tissue in response to allergen is their co-localisation with CD11c⁺ cells, a phenomenon that
306 occurs before subsequent allergen exposure ([Figure 1](#)). Given the reduction in the Th2
307 response, we conclude that the actions of platelets during allergen-sensitization are probably
308 delivered via an IgE-dependent FcεRI process to interact with lung APCs, for example CD11c⁺
309 DCs to process allergen signalling with T-cells in bronchial lymph nodes, although the data in
310 this manuscript are restricted to CD11c⁺ cells, rather than specifically DCs that express the
311 CD11c antigen. We note that in contrast to other studies,²⁴ we do not observe effects of
312 platelet depletion on IgE and Th2 cytokine production after allergen exposure, either acutely (3
313 days of allergen exposure) or chronically (8 weeks of allergen exposure), and the effects of
314 platelets on Th2 cytokine production are constrained temporally to the time of allergen
315 sensitization.^{3,13,25}

316 The phenomenon that allergen-exposed platelets are necessary for pulmonary CD11c⁺ cell
 317 recruitment after secondary allergen exposure was unlikely to be due to an inability of the
 318 restored platelet population to efficiently recruit CD11c⁺ cells from the circulation, as our
 319 previous studies have revealed platelets from non allergen-sensitized mice retain their ability to
 320 recruit leukocytes.³ Furthermore, neutrophil recruitment to the lungs was not influenced by
 321 platelet depletion at the time of allergen sensitization, even though it is well understood that
 322 pulmonary neutrophil recruitment is dependent on intravascular interactions with platelets.^{10,}
 323 ²⁶

324 It remains to be understood why professional APCs such as DCs might require platelet
 325 interactions for a more efficient processing of allergen *in vivo*. DCs also express FcεRI in
 326 different conformations with cross-linked IgE, yet it has been reported that IgE-mediated
 327 activation of DCs in allergic Th-2 type inflammation might be immune-regulatory rather than
 328 pro-inflammatory.^{27,28} Clearly, IgE/FcεRI-mediated antigen presentation by DCs is considered to
 329 be a critical event in the priming of Th2-type cells,²⁹ but recent evidence suggests that this
 330 response is dependent on low antigen concentrations, rather than receptor signalling *per se*.³⁰ T
 331 cell activities induced after IgE/FcεRI dependent processes by DCs have been reported not to
 332 develop as efficiently into Th2-type effector cells than those generated via an IgE/FcεRI
 333 independent process.³¹ How DCs process antigen and elicit a Th-2 type response is complex,
 334 and might therefore involve other cell types, including platelets that have been implicated in
 335 the innate response and express FcεRI, CD23, and other Immunoglobulin receptors, as well as
 336 toll-like receptors (TLRs).² Indeed, it has been reported that platelet communication with APCs
 337 is regulated by CD40/CD40L interactions, and this might be an important component of DC

communication with T cells in the draining lymph nodes after platelet activation by allergen.^{32,33}

Currently, our understanding as to why APCs might require platelets to effect an immune response is incomplete, but perhaps platelet activation by allergen or pathogens via IgE-dependent processes is a more direct, innate response, with less regulation and control. Two recent studies report the ability of platelets to modulate the immune response in mouse models of eosinophilic and allergic airway inflammation.^{34,35} The transfer of human platelets restored papain-induced airway eosinophilia on an IL-33 deficient background in mice, supporting a hypothesis that platelets are important in the IL-33 dependent provocation of Th2 immune cells.³⁴ Furthermore, the platelet derived Wnt antagonist Dickkopf-1 was reported to be important for Th2 cell cytokine production in a house dust mite (HDM) model of asthma.³⁵ The pathways by which platelets might affect sensitization to allergens in patients with asthma has not yet been described, but it is of interest that platelets from patients allergic to *Dermatophagoides pteronyssinus* have been shown to be activated directly by the specific allergen, and not activated by other known allergens to which the allergic blood donor was not sensitized to.^{16,18} Thus, clinical data is suggestive of a interaction between platelets and the sensitization process.

It is not understood how induced expression of FcεRI occurs on circulating platelets (**Figure 2**). The percentage of platelets that express these receptors remains a small subset of the whole platelet population, which might suggest that megakaryocytes residing in the bone marrow are capable of differentiation to ultimately determine the phenotype of subsets of platelets. It is

interesting to note however, that this increase in platelet FcεRI expression is suppressed in mice subjected to the temporary thrombocytopaenia at the time of allergen sensitization (**Figure 2**). The lifespan of murine platelets is between 3-4 days, yet the recording was taken when the platelet population had fully recovered, suggesting that there is an establishment of immune memory in a subset of megakaryocytes to continue producing a population of platelets with increased expression of FcεRI.

To conclude, the initial processing of antigen in naive animals requires the involvement of platelets, and leads to an increased production of allergen responsive platelets perhaps transmitted from megakaryocytes during thrombopoiesis, and supports earlier clinical data reporting the ability of platelets to become activated by specific allergens to which individual patients with allergy become sensitized to.^{16,18,36} These platelets respond by recruiting to organs that are exposed to the external environment and migrate to prime resident APCs for an efficient antigen-dependent immune response after subsequent exposure to allergen.

Figure Legends

Figure 1. Association of platelets with CD11c⁺ cells in lungs of allergen-sensitized mice. Lungs from either sham-sensitized or allergen sensitized (OVA) mice were collected at 0, 24, or 48 hours after exposure to aerosolised OVA from day 15. Lung sections were stained for the presence of CD41⁺ (platelets, red fluorescence) and CD11c⁺ cells (green fluorescence): Representation of CD11c⁺ cell (white arrow) (A); platelets and CD11c⁺ cells localised to airways in sham sensitized (B) and allergen-sensitized mice (C). Arrows indicate presence of CD11c⁺ cells. A 'zoomed' image (using Microsoft Office) represented in the dotted oblong (C) shows individual platelets (red) attached to the CD11c⁺ cell (D). Images photographed using a x40 objective under a Zeiss Axiovert microscope. Images A-D were enhanced to improve brightness/contrast using Microsoft Powerpoint. The presence of CD11c⁺ cells was quantified around airway walls (E). The percentage of CD11c⁺ cells co-localized with CD41⁺ platelets was determined within the airway walls (F). n=4 per group. **P< 0.01, *P< 0.05 compared to sham control group.

Figure 2. Induction of temporary thrombocytopaenia during the period of allergen sensitization and effects on platelet FcεRI expression. Mice were administered either a control IgG antibody, or platelet-depleting anti-GPIIb/IIIa antibody on days -1 and 6 of the sensitization procedure to deplete circulating platelets within the time frame of allergen sensitization (OVA) on days 0 and 7 (A). Tail vein bleeds were conducted on days -1, 0, 9, and 14 to enumerate

406 circulating platelet and leukocyte numbers (A). Representative histograms show CD41+
 407 platelets from lysed whole blood (B), FcεRI expression in sham-sensitized (C), OVA-sensitized +
 408 IgG (D), and OVA-sensitized + anti-GPIbα mice (E). Expression recorded as % of cells expressing
 409 antigen on day 15 in the absence of secondary allergen exposure (F). n=4-5 per group. Data:
 410 means ±SEM. **P< 0.01 versus sham-sensitised control group, ##P<0.01 versus OVA-sensitised
 411 IgG group.

412

413 **Figure 3. Effects of temporary thrombocytopaenia during the period of allergen sensitization**
 414 **on the inflammatory response after subsequent allergen exposure.** Mice were administered
 415 either a control IgG antibody, or platelet-depleting anti-GPIbα antibody on days -1 and 6 of the
 416 sensitization procedure to deplete circulating platelets within the time frame of allergen
 417 sensitization (OVA) on days 0 and 7. On days 15, 16, and 17, mice were subsequently exposed
 418 to aerosolized allergen (OVA) for 30 mins/day before BAL was performed on day 18 and
 419 enumerated for eosinophils (B), macrophages (C), neutrophils (D), and lymphocytes (E). n=8-12
 420 per group. Data: means± SEM. *P< 0.05, **P< 0.01, ***P< 0.001 versus sham-sensitised control
 421 group. #P< 0.05, ##P< 0.01, versus OVA-sensitised group (OVA+ IgG).

422

423 **Figure 4. Effects of temporary thrombocytopaenia during the period of allergen sensitization**
 424 **on the cytokine expression and IgE titre after subsequent allergen exposure.** Mice were
 425 administered either a control IgG antibody, or platelet-depleting anti-GPIbα antibody on days -1
 426 and 6 of the sensitization procedure to deplete circulating platelets within the time frame of

allergen sensitization (OVA) on days 0 and 7. On days 15, 16, and 17, mice were subsequently exposed to aerosolized allergen (OVA) for 30 mins/day before BAL and blood was harvested on day 18 for plasma IgE analysis (A), and BAL enumerated for IL-4 (B), IL-5 (C), and IFN γ (D) titres. n=6-8 per group. Data: means \pm SEM. *P< 0.05 versus sham-sensitised control group. #P< 0.05 versus OVA-sensitised group (OVA+ IgG).

Figure 5. Effect of platelets and allergen on DC recruitment. Allergen-sensitized mice had previously been administered either control IgG or platelet-depleting anti-GPIIb/IIIa antibody during the allergen sensitization period, and lungs were harvested at 0 (day 15) and 24 hours after (day 16) exposure to aerosolized allergen (OVA) for histology or flow cytometry. Lungs were processed into a single cell suspension and CD11c⁺ cells were quantified via flow cytometry using fluorospheres to obtain an accurate count (A) at 0 hours (B), 24 hours (C) post allergen challenge. n=3-9. Data: means \pm SEM. ***P< 0.001 versus sham-sensitized group; and #P< 0.05 versus OVA-sensitized IgG group

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ONLINE SUPPLEMENTARY MATERIAL

Platelets play a central role in sensitization to allergen.

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A. Blood microsampling

For blood microsampling to enumerate circulating platelet and leukocyte numbers, mice were placed in a heated chamber (37 °C) for 10 minutes before a 1-2 mm cut was made with a scalpel blade across the lateral tail vein. Using a micropipette, 2 µl of blood was diluted 1:100 into stromatol solution for platelet counts, a further 2 µl diluted 1:50 in Turk's solution for leukocyte counts (both using a Neubauer improved haemocytometer). Manual counts were made using a Zeiss Axiovert microscope.

B. Immunohistochemistry of lungs.

At 0h, 24h, and 48h, post one day of allergen challenge on day 15, lungs were recovered from terminally anaesthetized mice. Lungs were inflated with a tracheal cannula with 4% PFA and then immersed to fix in 4% PFA for 1 hour. The lungs were then incubated in 10% sucrose solution (in PBS) overnight at 4°C. The sucrose solution was then replaced with 25% OCT (in PBS) for 1 hour, and then with 100% OCT for 1 hour. Lungs were then stored at -80°C, after which they were sectioned (6µm) with a Cryostat and placed on poly-L-lysine coated microscope slides. Tissue sections were stained with primary antibodies to detect CD11c+ cells (hamster anti-mouse CD11c Integrin α x chain, HL3, BD Biosciences) and

platelets (Goat polyclonal IgG Integrin α IIb. k-18, Santa Cruz Biotech), for 2 hours. Slides containing lung sections were then washed and incubated with secondary fluorescent antibodies: 1. Donkey anti-goat IgG-Cy3 for 1 hour, before PBS wash followed by 2. Goat-anti Armenian hamster IgG-FITC for 1 hour. Finally, slides were cover slipped using a photo bleaching protecting mountant and stored in the dark at 4°C until required for microscopic analysis.

C. Microscopic analysis and Lung morphometry

Lung section slides were analysed under a fluorescent microscope (Zeiss Axioskop) using a x100 objective, where CD11c+ cell localisation was measured around airway walls to a depth of 50 μ m beneath the sub-epithelial basement membrane, in a five field area. CD11c+ cells were investigated for platelet co-localisation (CD41+ events) and the extent of those with positive results quantified per airway using an image analysis programme (Scion Image; National Institutes of Health, Bethesda, MD).

D. Flow cytometric analysis of CD11c+ cells and platelets

All experiments were analysed on a flow cytometer (FC500 series Beckman Coulter) as previously described.^{10,16} Single cell suspensions isolated from lung digestion at 0 and 24 hours after allergen challenge on day 15 were incubated with the DC marker, anti-CD11c-fluorescein mAb, in a saturating concentration for 30 minutes at room temperature away from direct light. For quantification of CD11c+ cells, samples were also treated with Beckman coulter Flow CountTM fluorosphere beads (Beckman Coulter). Specific gating parameters were set up to calibrate for the assayed concentration of the fluorospheres to

allow absolute quantification of anti-CD11c-fluorescein positive events. For analysis of Platelet FcεRI expression, citrated blood was obtained via cardiac puncture on other groups of mice on day 15 in the absence allergen challenge (0 hours). Blood was incubated with platelet specific anti-CD41-phycoerythrin mAb and anti-FcεRI-fluorescein mAb in saturating concentrations for 30 minutes at room temperature and platelets were gated on size and FcεRI positive events were then measured on CD41 positive cells.

E. Quantification of IgE, IL-4, IL-5, and IFN-γ

Bronchoalveolar lavage samples taken at day 18 were centrifuged at 300 G for 5 minutes and supernatants harvested for quantification of IL-4, IL-5, and IFN-γ using specific ELISAs as per the manufacturer's instructions (Duoset, R&D systems). Total IgE was measured in plasma samples prepared from citrated blood harvested via cardiac puncture at day 18. A murine specific IgE ELISA was used as per the manufacturer's instructions (BD Pharmingen. Primary capture antibody: rat anti-mouse IgE clone R35-72).